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NOVEL METHOD FOR SYNTHESIS OF MELANIN BASED SILVER NANOPARTICLES AND ITS APPLICATION AGAINST STAPHYLOCOCCUS AUREUS

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ABSTRACT

A group of biopolymers is known as melanin, melanin is getting more attention due to its applications in diverse field such as cosmetics, bioremediation, and pharmaceutical industries. Melanin can be biosynthesized via bacteria and fungi. In present study melanin producing fungal strain was procured from repository and Production of melanin was carried out by submerged fermentation using tyrosine casein medium. Extraction and Characterization of melanin was carried out using various test like bleach test, solubility test, reaction with ferric chloride, reaction with hydrogen peroxide, thin layer chromatography and FTIR spectroscopy. Extracted melanin was used for synthesis of silver nanoparticles. Colour change after incubation of melanin with silver nitrate indicated formation of silver nanoparticles, further synthesized nanoparticles was characterized by UV-Visible spectroscopy, Dynamic light scattering and zeta potential analysis. Antibacterial activity of melanin based synthesized nanoparticles was found against Staphylococcus aureus. Melanin based synthesized nanoparticles can be proved as stable and eco friendly nature and having wide range of applications in fields like agriculture, textile, bioremediation, food coating, bio imaging, nanomedicine, and various pharmaceutical drugs.

KEYWORDS: Melanin, Nanoparticles, Nanomedicine. Bioimaging. Food Coating

INTRODUCTION

Melanin is a pigment formed by the oxidative polymerization of phenolic and indolic compounds. It is a natural pigment which have their presence in animals, birds, plants, humans, and in some species of the microorganisms. It has multifunctional activities, providing defence against environmental stresses such as Ultraviolet (UV) light, Oxidizing agents, and Ionizing radiation. Melanins are dark, generally black, biological macromolecules composed of various types of phenolic or indolic monomers, usually complexed with protein, and often with carbohydrates as well (Butler and Day, 1998). Melanin forms various groups of pigments synthesized in living organisms during hydroxylation and polymerization of organic compounds (Shrishailnath et al., 2010). Melanin ubiquitous pigments that are negatively charged, black amorphous pigments of high- molecular weight compounds (Nosanchuk et al., 2003). Melanin production has been reported by a variety of microorganisms such as *Magnaporthe grisea, Cryptococcus neoformans, Paracoccidioides brasiliensis, Sporothrix as schenckii, Aspergillus fumigates* (Langfelder et al., 2003), *Vibrio cholerae, Shewanella colwelliana, Alteromonas nigrifaciens (Soliev et al., 2011),* and many species of the genus Streptomyces (Manivasagan et al., 2013).

Some fungal melanin is found as part of the cell wall proper, generally recognizable as a distinct outermost and sharply defined layer, and some melanin is found in association with the fibrillar matrix which extends out from the cell wall of many fungi. These types of melanin are referred to as wall bound and are found in hyphal, conidial, and sclerotial walls. The wall bound melanins are reported in several fungi such as Thielaviopsis (Wheeler, 1979), chlamydospores of *Verticillium* (Wheeler, 1982), *Aureobasidium* (Lingappa, 1963), *Coccosporium* (Durrel, 1964), *Humicola* (Ellis et al., 1974), *Nadsoniella* (Ruban, 1969) walls of rind cells of sclerotia of *Botrytis cinerea* (Zeun, 1985).

Four basic types of melanin:

1. Eumelanin,
2. Pheomelanin,
3. Allomelanin,
4. Neuromelanin

Biosynthesis Pathway of Melanin

Two pathways of melanin synthesis are found in fungi.

1. Many fungi synthesize melanin via the DHN pathway. Polymerization of DHN leads to formation of melanin (Butler and Day 1998a; Langfelder et al. 2003).

2. A few fungi synthesize melanin via l-3,4-dihyroxyphenylalanine (l-dopa), in a pathway that resembles mammalian melanin biosynthesis. Tautomerization of dopachrome forms dihydroxyindoles that polymerize into melanin (Land et al. 2004; Langfelder et al. 2003;)

It is noteworthy that in both melanin pathways are found many fungi such as Sporothrix schenckii (Almeida-Paes et al. 2009), while others such as C. neoformans rely exclusively on the l-dopa pathway.

Nanoparticles

Nanoparticle the word nanoparticle for the nanoscale material.

Which are atomic or molecular or macro molecular aggregates with at least single dimension at least less than 100 nm. The physical and chemical properties of a nanoscale material is much effective than their bulk form. Nanoparticle are differed in morphologies like spherical, cylindrical, tubular etc (Margi Patel, et. al, 2017).

Nanoparticles are divided into two groups

1. Organic nanoparticle.
2. Inorganic nanoparticle.

liposome, polymerases, polymer constructs and micelles are the most widely studied organic nanoparticle. But inorganic nanoparticle is receiving much attention of a researchers because of their unique size dependent physiochemical properties. Physical such as magnetic and optical properties and chemical properties include stability and functionalization. Hybrid organic and inorganic nanoparticle achieving much attention for developing novel nanocomposite material and they have a novel property such as catalytic activities, optical properties.

The size range of nanomaterials approximately less than 100 nm can easily infuse the cell. Those have size about less than 50 nm can enter the most of the cell and those nanomaterials size about less than 20 nm can easily enter blood vessels to tissue, and through leap enter blood brain barrier. Nanoparticle are used as an imaging and therapeutic application. They have beneficial factors 1. high surface area to volume ratio 2. possibility of ubiquitous tissue accessibility. Inorganic nanoparticles magnetic nanoparticles or MPs are one of the most profound inorganic nanoscale materials. They also have a magnetic core maghemite. Cobalt and nickel are limited used due to their toxicity. Human have cationic metal nanoparticle (iron) in their body. These cationic iron present in the endosomes.

Biosynthesis of Metal Nanoparticles By Microorganisms

Synthesis of metal nanoscale material biological source are extremely used in recent years. Biological method is advantageous due to the eco-friendly, economical, and versatile also much easier to produce nanoparticle synthesis. Plant extract and microorganisms are used for the synthesis of metal nanoparticle. Capping and stabilizing agent are not used in the biobased synthesis. The synthesis of nanoparticle using photogenic or myogenic approach. Both have their advantages as well as disadvantage. Photogenic approach is time consuming and relatively simpler. In photogenic approach phytochemical such phenol, flavonoid, terpenoids leads to produce Polydisperse nanoparticle. Biosynthesis by microorganism is much eco-friendly and avoid the use of a harmful chemicals. Microorganisms do not have high energy consumption and environment (Mohammad ovais *et.al,* 2018).

Mycosynthesis of Nanoparticle

Biosynthesis of different metal nanoparticle mycological approach have been successfully applied. In fungi, like bacteria extracellular and intracellular mechanism are used for the synthesis of metal nanoscale material. In intracellular approach fungi mycelium used metal salt and converted into less toxic material. Fungal extract is used in the extracellular approach. Fungi are much effective than the bacterial cell due to the presence of bioactive metabolite, high accumulation and enhance production (Mohammad ovais *et.al,* 2018).

The fungal mediated green chemistry approach towards the fabrication of NPs has many advantages. This includes easy and simple scale up method, economic viability, easy downstream processing and biomass handling, and recovery of large surface area with optimum growth of mycelia (Sastry et al., 2003). It has been observed that most of the fungal genera are coupled with the synthesis of Ag NPs either intracellularly or extracellularly showing the onset of deep brown coloration (Sastry et al., 2003). Aqueous Ag ions exposed to *Fusarium oxysporum* leads to the fabrication of extremely stable Ag hydrosol. The particles are in the 5-15 nm range and are stabilized in solution by the proteins excreted through the fungus (Ahmad et al., 2003a).

Characterization Tools For Nanoparticle

Nanoparticle have size range between 1 to 100 nm according to national Institute of health. Example of nanoscale material is Liposome, dendrimers, carbon, nanorod, carbon nanotubes, graphene, derivatives, titanium oxide, nanowire, silver nanoparticle, platinum nanoparticle, magnetic nanoparticle, quantum dots. Several methods are used for the characterization of nanoparticle such as NSOM, SEM, TEM, STM, AFM, DLS, FCS, RS, CD, IR, NMR, MS, Zeta potential, XRD, SAXS and other method include UV vis spectroscopy, FS (Lin et.al, 2014).

MATERIALS AND METHOLOGY

Procurement of fungal isolate

Fungal isolate was procured from central repository of Shrimad Rajchandra Vidyapeeth Dharampur And stored on agar slant till further process.

Morphological characteristics of fungal isolate

Tease mount method is used for mounting of fungi. Isolate colonies were picked from tyrosine casein agar medium and observed under microscope at 40x. And in it, filamentous growth and septate hyphae were observed against a blue background (Vacharavel shamly et al., 2014).

Production of Melanin pigment by selected isolate

Pigment production was carried out in Tyrosine casein broth respectively, through the Submerged fermentation (SF) method. After incubation period of 5-7 days at room temperature. Colour change from yellow to black indicates melanin synthesis (*Vacharavel shamly et al., 2014*) (Figure 6).

Extraction of melanin pigment

After the incubation medium was centrifuged at 10000 rpm for 10 minutes. The pH of the supernatant was adjusted to 10 with 5 M NaOH and then adjusted to pH 2 with 5 M HCl. This mixture was again centrifuged to obtain the melanin crude extract. Then equal volume of organic solvent- Chloroform: Ethyl acetate: Acetone was added in 1:1:1 ratio and mixed to remove proteins. An aqueous phase was collected by two phase separation using separating funnel. Then, aqueous phase was

collected and kept for drying overnight. Weighted it and stored at refrigerator for further study (*Gaber et al., 2020*).

Characterization of melanin pigment

All the following tests were carried out with melanin solution (dissolved in water; 1ml: 0.1gm) and Chemicals, Reagents, Solvents also in 1 ml.

Bleach test

For the analysis of bleaching reaction, slide test was carried out, in which crude melanin solution was kept on a clean slide and diluted solution of Potassium permanganate ($KMnO_4$) was added onto it. Potassium permanganate serves as bleaching agent which bleaches the melanin and turns the brown colour of pigment to colourless (*Hongwu sshen et al., 2015*).

Solubility test

The solubility test was carried out by checking solubility in different solvents. The solubility of melanin solution in Distilled Water, tap water, 0.1 N HCl, 5 M NaOH, 0.1 N NaOH, 5 M HCl, Chloroform, Ethyl acetate, Acetone, Butanol, Glacial acetic acid was determined (*Fangdong Zhan et al., 2011*).

Reaction with Ferric chloride/Iron chloride ($FeCl_3$)

The reaction of melanin solution and $FeCl_3$ solution was checked in a test tube and a colour change was observed (*Fangdong Zhan et al., 2011*).

Reaction with Hydrogen peroxide (H_2O_2)

The reaction of melanin solution and Strong Oxidizing agent H_2O_2 also recorded in test tube. solubility and colour change also observed in it (*Fangdong Zhan et al., 2011*).

Thin Layer Chromatography (TLC)

The pigment was separated using ready-made silica gel coated on alumina sheets. The separation was carried out using organic solvents such as n-butanol, glacial acetic acid and water in the ratio of 12:3:5. The chromatoplate was uniformly sprayed with ninhydrin reagent. Then the chromatogram was heated in a hot air oven at 105 °C for 4 min. In last the RF value was calculated (*Rakesh Patel et.al.,2017*).

Spectroscopy (FTIR) analysis of Melanin pigment

Fourier transform infrared spectroscopy (FTIR) is most useful for identifying the functional groups and interpretation of structure of unknown compounds (*EL-Ahmady et al., 2017*). Melanin pigment analysis was carried out at Centre of Excellence, Vapi.

Synthesis of Melanin-based Silver Nanoparticles

1 g of extracted melanin is added to 100 ml of D.W. and mixed, then 1 mM $AgNo_3$ is added and mixed and placed on an environmental rotary shaker for 24 hours. And taken O.D. (*Kiran et al., 2014*).

Characterization of synthesized silver nanoparticles

UV-Spectroscopy

UV-visible spectra were recorded as a function of wavelength using UV-vis spectrophotometer from 380-680 nm. Formation of silver nanoparticles can be easily detected by spectroscopy because the silver nanoparticles show atypical absorbance peak near 400 nm (*Nafisha Patel*et.al., 2018*).

Particle Size Distribution

Dynamic light scattering which is based on laser diffraction method with multiple scattering techniques was employ to study the average particle size distribution of silver nanoparticles. DLS were performed of synthesized nanoparticles for determination of average size distribution of particles using Malvern software (*Nafisha Patel*et.al., 2018*).

Zeta Potential

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles and is one of the fundamental parameters known to affect stability. Its measurement brings detailed insight into the causes of dispersion, aggregation or flocculation, and can be applied to improve the formulation of dispersions, emulsions and suspensions. Malvern Paralytical offers leading zeta potential analyzers for the measurement of zeta potential, or electrophoretic mobility (*Xu-yu Zhang et al., 2020*).

Antimicrobial Application of melanin based synthesized silver nanoparticles

Distilled water, silver nitrate solution, melanin-based silver nanostructures and melanin solution combination were tested for antimicrobial activity using well diffusion method and halo area was measured. The synthesized nanostructures were tested against common food pathogens (Gram positive and Gram negative) such as Staphylococcus aureus, Salmonella typhi, Proteus vulgaris and Bacillus megaterium. These were cultured in nutrient broth (HI media). Wells were made with a sterile steel cork borer and 100 micro-litterers of distilled water, silver nitrate solution, melanin solution and melanin-based silver nanostructures were added to the wells, incubated at 37°C for 24 h. After incubation the apparent halo was measured and the area of inhibition in mm was calculated (Kiran et al., 2014).

RESULTS AND DISCUSSION

Morphological characteristics of fungal isolate

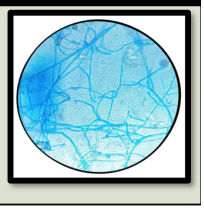
Sample	Morphological characteristics	Figure
Fungal isolate procured from Shrimad Rajchandra Vidyapeeth Dharampur	filamentous growth and septate hyphae observed against blue background	

Figure 1: Morphological characteristics of fungal isolate

Production of Melanin pigment by fungal isolate Pigment production was carried out in Tyrosine casein broth respectively, through the Submerged fermentation (SF) method. After incubation period of 5-7 days at room temperature. Colour change from yellow to black indicates melanin synthesis

Figure 2: Melanin production appear in Flask A and Flask B

Extraction of melanin pigment

It was suggested that melanin polymers constitute the building blocks of melanin granules. the process of granules formation and their dimension are strongly pH dependent, where a low pH promotes the aggregate growth and a high pH induces the breakup of the granules to small particles-oligomers with a lower degree of polymerization. this process is a consequence of the polyelectrolyte nature of melanin, and it is dependent on the ionization state of melanin groups like carboxylic, phenolic, and aminic groups as well as on the ionic strength of the environment (Gaber et al., 2020).

After 5 days of incubation melanin was extracted and yield 0.421gm/50 ml was obtained. The extracted pigment shows in figure

Figure 3: A. Two phase separation using separating funnel, B. Dried crude extract obtained (0.421gm/50 ml)

5.4 Characterization of melanin pigment

Bleach test

Potassium permanganate (KMnO4) has been used as a bleaching agent, which is economical as well as non-polluting. When melanin is present in large amounts; cell details may be obscured. Also, ability to be bleached serves as an identifying factor for melanin. The bleaching of excessive melanin helps in diagnosis of ocular tumours based on histological reports (Pillaiyar et al., 2017). In the experiment conducted, when crude melanin was subjected to dilute KMnO4 solution, bleaching of melanin was observed as brown colour of melanin turned colourless due to the action of strong oxidising agent. Though the use of KMnO4 as a bleaching compound is harmful and restricted in certain countries, it is still being used in combination with Oxalic/ Sulphuric acid.

Figure 4: result of bleach test

Solubility test

Melanin was soluble in various solution such as, distilled water, tap water, 0.1N HCl, 0.1N NaOH, 5M HCl, 5M NaOH, Ethanol. Melanin was insoluble in solvents like, ethyl acetate, glacial acetic acid, acetone, butanol, chloroform (*Fangdong Zhan et al., 2011*) (table 2).

Solution / Solvent	Solubility reaction
Distilled water, Tap water, 0.1 N NaOH, 0.1 N HCl, 5 M NaOH, 5 M HCl, Ethanol	Soluble
Ethyl acetate, glacial acetic acid, acetone, butanol, chloroform	Insoluble

Table 1: Solubility reaction of melanin pigment in different solution/ solvent

Reaction with Ferric chloride/Iron chloride ($FeCl_3$)

When extracted melanin was reacted with FeCl3 it gives brown color with $Fecl_3$ (*Fangdong Zhan et al., 2011*) (figure 9).

Reaction with Hydrogen peroxide (H_2O_2)

The reaction of melanin solution and Strong Oxidizing agent H_2O_2 also recorded in test tube. solubility and colour change also observed in it (*Fangdong Zhan et al., 2011*).

Thin Layer Chromatography (TLC)

TLC is a type of purification and separation technique. Separation was done using Butanol: Glacial Acetic acid: water (12:3:5) ratio. After the chromatogram was generated, separated spots of pigment was calculated. Retention factor (Rf) value of the spot of melanin pigment was calculated to be 0.6 which is near to the analysis of melanin done by Diraviyam et at.,2020.

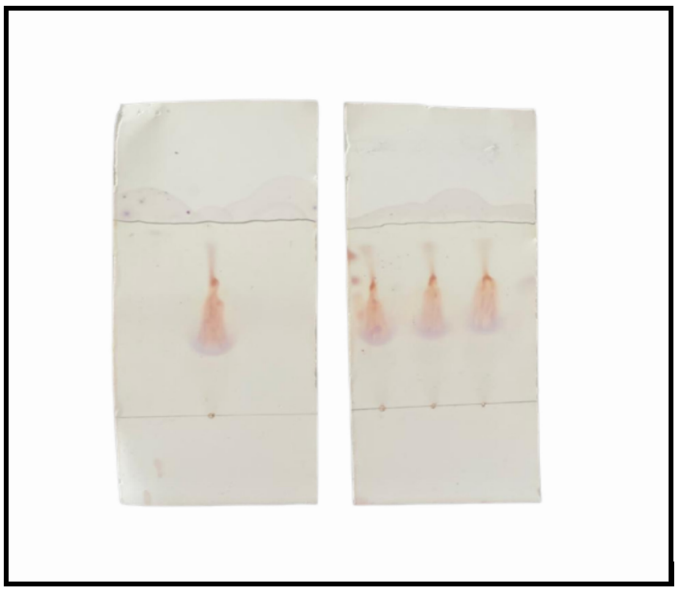
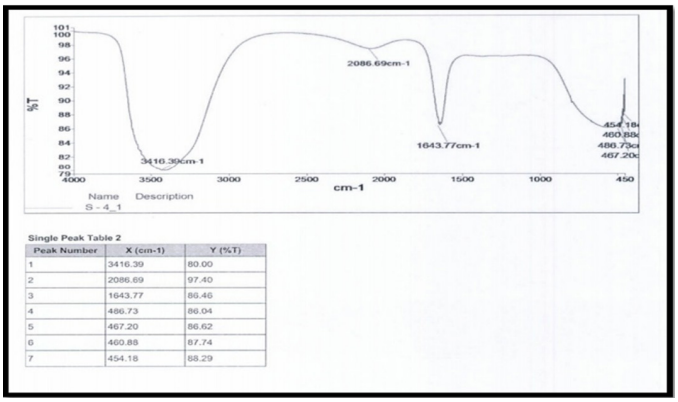


Figure 5: Result of TLC of the melanin pigment.

Spectroscopy (FTIR) analysis of Melanin pigment The FTIR spectrum of the extracted melanin show (figure 11) a strong, broad peaks around 3416.39 cm^{-1} correspond to the O-H group. Medium band at 2086.69 cm^{-1} can be assigned to stretching vibration of strong N=C=S isothiocyanate group. Peak observed around 1643.77 cm^{-1} are attributed to binding of C=C alkene group (*EL-Ahmady et al., 2017*).



Peak Number	X (cm-1)	Y (%T)
1	3416.39	80.00
2	2086.69	97.40
3	1643.77	86.46
4	486.73	86.04
5	467.20	86.62
6	460.88	87.74
7	454.18	88.29

Figure 6. FTIR spectra of extracted crude melanin

Synthesis of Melanin-based Silver Nanoparticles
Colour changes from pale to brown after 24 hours indicates formation of silver nanoparticles.

Before incubation After incubation

Figure 7: Result of synthesis of melanin-based silver nanoparticles

Characterization of synthesized silver nanoparticles

UV-Spectroscopy

The synthesized nanoparticles were primarily characterized by UV- vis spectrophotometer. The UV-vis spectra of synthesized nanoparticles were recorded. The strongest peak was obtained at 420 nm which is typical absorbance peak of silver nanoparticles. There was increase in absorbance with the increase in incubation time. The microbially synthesized nanoparticles from all the cultures showed a maximum absorption at 420nm.

Particle Size Distribution

Particle size distribution of synthesized silver nanoparticles was carried out by dynamic light scattering. figure show the particles size distribution of silver nanoparticles synthesized by melanin Z (d. nm) average found to be 655.7 nm.

The same type of result of result were also found by (Nafisha Patel et.al., 2018) And (Xu-yu Zhang et al., 2020).

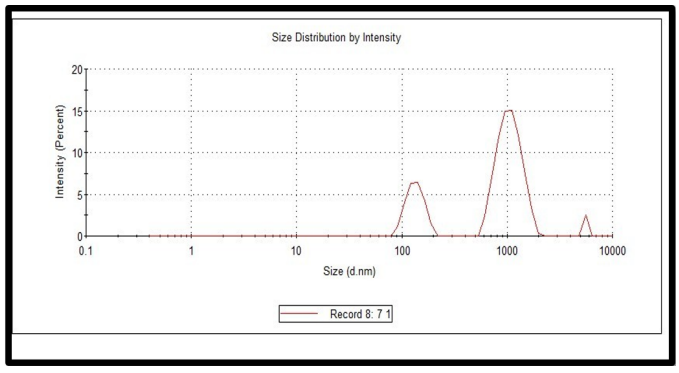


Figure 8: Particle size distribution of silver nanoparticles

Zeta Potential

If all the particles in suspension have a large negative or zeta potential, then they will tend or repel each other and there is no tendency to flocculate. It can be interpreted that silver nanoparticles biosynthesized from melanin has zeta potential value around -6.26 mV.

Antibacterial activity of Melanin-based Silver Nanoparticles
The melanin-based silver nanoparticles showed antimicrobial

activity against many food pathogens tested but the highest activity was found against S. aureus (17 mm).

The same type of work done by Kiran et al., 2014 and they found that melanin-based silver nanoparticles showed antibacterial activity.

Test	Zone diameter(mm) against *Staphylococcus Aureus*
D/W	0
Melanin	0
Silver nitrate	0
Silver nanoparticles	17

CONCLUSION

According to the results shown above, that the pigment produced from the isolate fungi is successfully occurred. Pigment production was carried out in Tyrosine casein broth, through the Submerged fermentation (SF) method. After incubation period of 5-7 days at room temperature Colour change from yellow to black indicates melanin production. 0.421gm/50 ml melanin yield was obtained. Characterization of the extracted melanin was done by different test. The extracted melanin was used for synthesis of melanin-based silver nanoparticles. Further characterization of melanin-based silver nanoparticles was done by UV- Vis spectroscopy, Particle size distribution and Zeta potential. Melanin-based silver nanoparticles were applied on pathogenic bacteria which showed broad-spectrum antimicrobial activity against pathogenic bacteria.

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